

METHODS OF TREATING PIN1 ASSOCIATED DISORDERS BY COVALENT MODIFICATION OF ACTIVE SITE RESIDUES

Related Applications

5 This application claims priority to U.S. Provisional Application No. 60/463,810
filed April 17, 2003, the entire contents of which are incorporated herein by reference.
This application is further related to U.S. Application 10/XXX,XXX, entitled
“*PHOTOCHEMOTHERAPEUTIC COMPOUNDS FOR USE IN TREATMENT OF*
PIN1-ASSOCIATED STATES,” filed on even date herewith, the entire contents of
10 which are incorporated herein by reference.

Technical Field of the Invention

 This invention relates to methods for treating disorders associated with Pin1 or
that are related to Pin1. The methods focus on treating these disorders with
15 modulators, e.g., inhibitors, of the activity of Pin1 or Pin1 related polypeptides.

Background of the Invention

 The peptidyl-prolyl cis-trans isomerases (PPIases), or rotamases, are a family
of ubiquitous enzymes that catalyze the cis/trans isomerization of the peptide bond on
20 the n-terminal side of proline residues in proteins (Hunter, Cell 92:141-142, 1998).
PPIases are divided into three classes, cyclophilins (Cyphs), FK-506 binding proteins
(FKBPs) and the Pin1/parvulin class.

 Cyclophilins and FKBPs are distinguished by their ability to bind the
clinically immunosuppressive drugs cyclosporin and FK506, respectively (Schreiber,
25 Science 251:283-7, 1991; Hunter, *supra*). Upon binding of these drugs, there are two
common outcomes: inhibition of the PPIase activity and inhibition of the common
target calcineurin. The inhibition of calcineurin phosphatase activity prevents
lymphocytes from responding to antigen-induced mitogenic signals, thus resulting in
immunosuppression. However, the inhibition of the PPIase activity is apparently
30 unrelated to the immunosuppressive property of the drug/PPIase complexes. Even
more surprisingly, deletion of all 8 known cyclophilins and 4 FKBPs in the same cells
does not result in any significant phenotype (Dolinski et al., Proc. Natl. Acad. Sci.
USA 94:13093-131098, 1997).

In contrast, members of the Pin1/parvulin class of PPIases bind neither of these immunosuppressive drugs, and are structurally unrelated to the other two classes of PPIases. Known members of the Pin1/parvulin class include Pins1-3 (Lu et al., Nature 380:544-547, 1996), Pin-L (Campbell et al., Genomics 44:157-162, 1997),
 5 parvulin (Rahfeld, et al., Proc. Natl. Acad. Sci. USA 93:447-451, 1996) and Ess1/Pft1 (Hanes et al., Yeast 5:55-72, 1989; and Hani, et al. FEBS Letts 365:198-202, 1995).

Pin1 is a highly conserved protein that catalyzes the isomerization of only phosphorylated Ser/Thr-Pro bonds (Rananathan, R. *et al.* (1997) *Cell* 89:875-86; Yaffe, et al. 1997, Science 278:1957-1960; Shen, et al. 1998, Genes Dev. 12:706-720;
 10 Lu, et al. 1999, Science 283:1325-1328; Crenshaw, et al. 1998, Embo J. 17:1315-1327; Lu, et al. 1999, Nature 399:784-788; Zhou, et al. 1999, Cell Mol. Life Sci. 56:788-806). In addition, Pin1 contains an N-terminal WW domain, which functions as a phosphorylated Ser/Thre-Pro binding module (Sudol, M. (1996) *Prog. Biophys. Mol. Biol.* 65:113-32). This phosphorylation-dependent interaction targets Pin1 to a
 15 subset of phosphorylated substrates, including Cdc25, Wee 1, Myt1, Tau-Rad4, and the C-terminal domain of RNA polymerase II large domain (Crenshaw, D.G., *et al.* (1998) *Embo. J.* 17:1315-27; Shen, M. (1998) *Genes Dev.* 12:706-20; Wells, N.J. (1999) *J. Cell. Sci.* 112: 3861-71).

The specificity of Pin1 activity is essential for cell growth; depletion or
 20 mutations of Pin1 cause growth arrest, affect cell cycle checkpoints and induce premature mitotic entry, mitotic arrest and apoptosis in human tumor cells, yeast or *Xenopus* extracts (Lu, et al. 1996, Nature 380:544-547; Winkler, et al. 200, Science 287:1644-1647; Hani, et al. 1999. J. Biol. Chem. 274:108-116). In addition, Pin1 is dramatically overexpressed in human cancer samples and the levels of Pin1 are
 25 correlated with the aggressiveness of tumors. Moreover, inhibition of Pin1 by various approaches, including Pin1 antisense polynucleotides or genetic depletion, kills human and yeast dividing cells by inducing premature mitotic entry and apoptosis.

Thus, Pin1-catalyzed prolyl isomerization regulates the conformation and function of these phosphoprotein substrates and facilitates dephosphorylation because
 30 of the conformational specificity of some phosphatases. Thus, Pin1-dependent peptide bond isomerization is a critical post-phosphorylation regulatory mechanism, allowing cells to turn phosphoprotein function on or off with high efficiency and specificity during temporally regulated events, including the cell cycle (Lu *et al.*, *supra*).

Recent crystal structure data has elucidated the geometry and location of the Pin1 active site. Based on the information obtained from these data, e.g., the location and identity of residues present in the active site, specific covalent and non-covalent modulators of Pin1, and polypeptides that are structurally and functionally related to Pin1 can be designed.

Taken together, these results indicate that Pin1 and the subfamily of polypeptides that are related to Pin1 are a novel target for diseases characterized by uncontrolled cell proliferation, primarily malignancies. Therefore, there is an ongoing need for specific inhibitors of Pin1 and Pin1-related proteins, and for reliable methods of designing such inhibitors.

Summary of the Invention

The present invention provides methods for treating a subject suffering from a Pin1 associated disorder or a PRTP disorder.

In one embodiment the invention pertains to a method of treating a Pin1 associated disorder, or a PRTP disorder, by administering a MSPCIT to a subject. In addition to a moiety that covalently interacts with Pin1, or a PRTP, the MSPCIT can contain any one or more of the following moieties:

A-B-C-D-E

where A is a hydrophobic pocket interacting moiety; where B is a cysteine/serine valley interacting moiety; where C is a phosphate pocket interacting moiety; and where D is a substrate entry groove interacting moiety and where E is a lip region interacting moiety such that administration of any combination of these moieties treats the Pin1 disorder. The MSPCIT can be any of A-B-C-D-E moieties, or a moiety that is not classified as any of the A-B-C-D-E moieties.

In related embodiments, the MSPCIT of the invention can contain any one, two, three, four or five of the interacting moieties and these moieties can be covalently linked. The moieties can be small molecules, peptides or peptidomimetics.

In a related embodiment, the MSPCIT can interact with any residue that is capable of being covalently modified, e.g., cysteine or serine, in the active site. In a specific embodiment, the MSPCIT can interact with cysteine-113 of Pin1. In another

specific embodiment, the MSPCIT can interact with serine-114 of Pin1. This interaction can be through, for example, a disulfide bond or a Michael adduct.

In another embodiment, the invention pertains to novel compositions that contain moiety that is able to specifically interact with a Pin1, or a PRTP, active site residue through a covalent interaction. These molecules may contain, in addition to the covalently interacting moiety, a moiety that interacts with anyone or more of the following areas of the active site: the phosphate pocket, the hydrophobic binding pocket, the serine/cysteine valley, the substrate entry groove, and the lip region.

Brief Description of the Figures

Figure 1 depicts the amino acid sequence of Pin1 (SEQ ID NO:1).

Figure 2 depicts the results of a time dependant inactivation of Pin1 by juglone and Fred-A. The graph depicts the observed rate constant as a function of time (in minutes) for Pin1 alone, Pin1 and 100 uM juglone, Pin1 and 200uM fred-A, and a control.

Detailed Description

Definitions

The term "MSPCIT" is intended to include modulators specific for Pin-1, or a polypeptide related to Pin1, that covalently interact with a target site. The term "target site" includes amino acid residues in the active site of Pin1, or a PRTP, that are amenable to covalent modification, e.g., cysteine or serine residues. The MSPCIT of the invention may include multiple moieties that are capable of interacting with regions of the active site defined herein. At least one moiety of the MSPCIT must covalently interact with a Pin1, or a PRTP, active site residue.

The term "interacting agent" includes substances which can interact with the Pin1 polypeptide such that the three-dimensional structure can be determined. In one embodiment, the interacting agent is a Pin1 inhibitor, a substrate-derived peptide, or a solvent based molecule.

The term "co-complex" refers to a Pin1 polypeptide, Pin1 related polypeptide or fragment thereof in covalent or non-covalent association with a chemical entity or compound.

The term "modulator of Pin1" are compounds that have the ability to modulate the activity of Pin1 (SEQ ID NO.:1), or compounds that have the ability to modulate the activity of PRTP. These modulators can be, for example, Pin1 inhibitors.

The term "Pin1 inhibitor" refers to any molecule that can interact with Pin1 or a Pin1-related polypeptide and inhibit the ability of the polypeptide to carry out proline isomerization activity. Compounds within the scope of the invention can be naturally occurring or chemically synthesized. The term is also intended to include pharmaceutically acceptable salts of the compounds. In certain embodiments, the inhibitor is specific for Pin1, *i.e.*, does not inhibit the isomerase activity of PPIases belonging to other classes (*e.g.*, cyclophilins or FKBP). In at least one embodiment, the Pin1 inhibitor is not Fredericamycin A or a Fredericamycin-related compound.

The term "Pin1-associated state" or "Pin1 associated disorder" includes disorders and states (*e.g.*, a disease state) which are associated with abnormal cell growth, abnormal cell proliferation, or aberrant levels of Pin1 marker. Pin1-associated states include states resulting from an elevation in the expression of cyclin D1 and/or Pin1. Pin1-associated states also include states resulting from an elevation in the phosphorylation level of c-Jun, particularly phosphorylation of c-Jun on Ser^{63/73}-Pro and/or from an elevation in the level of c-Jun amino terminal kinases (JNKs) present in a cell. Pin1-associated states include neoplasia, cancer, undesirable cell growth, and/or tumor growth. Pin1-associated states include states caused by DNA damage, an oncogenic protein (*i.e.* Ha-Ras), loss of or reduced expression of a tumor suppressor (*i.e.* Brca1), and/or growth factors.

The term "PRTP" is intended to refer to polypeptides related to Pin1. These polypeptides include polypeptide that are homologous to Pin1, polypeptide that are functional homologues of Pin1, *e.g.*, polypeptides that catalyze the cis/trans isomerization of the peptide bond on the n-terminal side of proline residues in proteins, or polypeptides that share sequence identity with Pin1. In one embodiment the PRTP polypeptide of the invention is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% identical to sequence of Pin1 set forth as SEQ ID NO:1. Exemplary PRTP molecules include cyclophilins (Cyphs) and FK-506 binding proteins (FKBPs).

The term "PRTP-associated state" or "PRTP-associated disorder" are intended to include any condition, disorder, or disease that is related to or caused by a polypeptide related to Pin1. PRTP disorders include those conditions, diseases, and disorders caused by polypeptides that are homologous to Pin1 or polypeptides that catalyze the isomerization of peptide bonds.

The language "Pin1 inhibited-state" is intended to include states in which one activity of Pin1, or a PTRP, is inhibited in cells, *e.g.*, cells in a subject, that have been treated with a Pin1 modulating compound. "Pin1 inhibited-state" is also intended to include states wherein the Pin1 modulating compound is administered to a subject, allowed to remain in a preactivated state, and subsequently activated by a stimulus. The stimulus may be selected from a natural event, artificial event, or the combination thereof. For example, the natural event may be the action of an enzyme and/or the artificial event may be the addition of a hyperplastic inhibitory agent or the addition of energy to the subjects system in any manner that achieves activation, *e.g.*, by radiation, *e.g.*, by light with a wavelength greater than about 400 nm, *e.g.*, greater than about 600 nm, *e.g.*, greater than about 620 nm, *e.g.*, greater than about 630 nm, *e.g.*, greater than about 640 nm, *e.g.*, greater than about 650 nm. In one embodiment, the cells enter a Pin1 inhibited-state for a designated period of time prior to activation of the modulating compound sufficient to allow the modulation the activity of Pin1 by the activated modulating compound. In certain embodiments of the invention, the designated period of time prior to activation is greater than about 1 hour, *e.g.*, greater than about 2 hours, *e.g.*, greater than about 3 hours, *e.g.*, greater than about 6 hours, *e.g.*, greater than about 12 hours, *e.g.*, greater than about 24 hours, *e.g.*, greater than about 36 hours, *e.g.*, greater than about 48 hours, *e.g.*, greater than about 72 hours. In a specific embodiment, the designated period of time prior to activation is 3 days. In one embodiment, the Pin1 modulating compound is preactivated prior to administration to a subject followed by the introduction of at least one stimulus sufficient to allow the modulation the activity of Pin1 by the modulating compound. In certain embodiment of the invention, the activity of the modulating compound is enhanced by the entrance of the cells, *e.g.*, cells of a subject, into a Pin1 inhibited state.

A "competitive" inhibitor as used herein is one that inhibits proline isomerase activity by binding to the same kinetic form of the enzyme as its substrate binds, thus

directly competing with the substrate for the active site. Competitive inhibition can be reversed completely by increasing the substrate concentration.

An "uncompetitive" inhibitor as used herein is one that inhibits proline isomerase activity by binding to a different kinetic form of the enzyme than does the substrate. Such inhibitors bind to the enzyme already bound with the substrate and not to the free enzyme. Uncompetitive inhibition cannot be completely reversed by increasing the substrate concentration.

A "noncompetitive" inhibitor as used herein is one that can bind to either the free or substrate bound enzyme.

The term "interaction template" refers to a three dimensional model built using information obtained from crystal structures solved with chemical entities bound in the active site. The interaction template is formed by using a Pin1 polypeptide, or a PRTP, having a substitution insertion or deletion of one or more amino acids of the amino acid sequence set forth in SEQ ID NO:1, wherein the active site of the Pin1 polypeptide is accessible to solvent and available for interaction with modulators, e.g., inhibitors. This crystallized form of Pin1 is used to facilitate solving high resolution molecular structures with molecules bound in the active site such that the residues capable of interacting with inhibitors in the active site are determined. This template is used in the design of high affinity inhibitors of Pin1 isomerase activity.

The term "specificity template" refers to a template created by comparing sequence alignments of homologous or functionally related proteins. Identification of conserved and non-conserved residues allows a skilled artisan to design inhibitors of prolyl isomerase activity that increase affinity and specificity. The determination of conserved amino acids allows the skilled artisan to develop inhibitors with increased affinity but not, necessarily, specificity. The determination of non conserved amino acids allows the skilled artisan to develop inhibitors that have increased affinity and specificity.

The term "hydrophobic pocket" refers to the portion of the active site that binds a hydrophobic moiety. In one embodiment, the hydrophobic pocket contains 4, 6, 8, 10, 12 or 14 hydrophobic amino acid residues. In one particular embodiment, the hydrophobic pocket contains amino acid residues His59, Leu61, Leu122, Phe125, Met130, Gln131, Pro133, Phe134, Thr152, and His157 of SEQ ID NO:1.

The term “cysteine/serine valley” refers to a portion of the active site that is responsible for binding the serine moiety of the substrate. In one embodiment this region contains residues Leu61, Cys113 and Ser154 of SEQ ID NO:1.

5 The term “phosphate binding pocket” refers to a region of the active site containing three positively charged amino acids that binds negatively charged moieties or hydrogen donor/acceptor groups. In one embodiment, this pocket is contains 4, 6, 8, or 10 amino acid residues. In one particular embodiment, this pocket is defined by residues Lys63, Ser67, Arg68, Arg69, Pro70 and Ser154.

10 The term “substrate entry groove” refers to a region of the polypeptide that allows for substrate entry into the active site. In one embodiment this groove contains amino acids Lys63, Arg69, Ser71, Ser72, Trp73, Arg74, Gln75, Glu76, Asp112, Cys113, Ser114.

15 The term “lip regions” refers to the residues that surround the active site cavity, as defined previously. In one embodiment these lip regions contain residues that are within 10 Å of the active site cavity. In one particular embodiment, this lip region is defined by, but not limited to, residues Arg54, Arg56, His64, Ser65, Gln66, Lys77, Ile78, Thr79, Ser115, Lys117, Ala118, Gly123, Ala124, Phe125, Ser126, Arg127, Gly128, Gln129, Pro133, Glu135, Lys132, Phe151, Asp153, Gly155, and Ile156.

20 The term “hydrophobic pocket interacting moiety” refers to a compound that is capable of interacting with the residues of the hydrophobic pocket. This interaction can be, for example, through hydrophobic interactions, through van der Waals contacts, or through hydrogen bonds.

25 The term “cysteine/serine valley interacting moiety” refers to a compound that is capable of interacting with Cys113 or Ser154, i.e., the cysteine/serine valley, within the region of the active site that binds the serine residue of the natural substrate. This interaction can be covalent, noncovalent, through hydrogen bonds, or by van der Waals interactions.

30 The term “phosphate binding pocket interacting moiety” refers to a compound that can interact with the phosphate binding pocket. This interaction can be electrostatic, through salt bridges, covalent, or through van der Waals interactions.

The term “substrate entry groove interacting moiety” refers to a compound that can interact with the substrate entry groove. This interaction can be electrostatic, hydrophobic, covalent, hydrogen bonding, or through van der Waals interactions

The term “lip region interacting moiety” refers to a compound that is capable of interacting with the residues outside the active site. This interaction can be, for example through hydrophobic interactions, though van der Waals contacts, or through hydrogen bonds.

5 The term "radiation therapy" includes the application of a genetically and somatically safe level of electrons, protons, or photons, both localized and non-localized, to a subject to inhibit, reduce, or prevent symptoms or conditions associated with undesirable cell growth. The term X-rays is also intended to include machine-generated radiation, clinically acceptable radioactive elements, and isotopes thereof,
10 as well as the radioactive emissions therefrom. Examples of the types of emissions include alpha rays, beta rays including hard betas, high-energy electrons, and gamma rays. Radiation therapy is well known in the art (see *e.g.*, Fishbach, F., *Laboratory Diagnostic Tests*, 3rd Ed., Ch. 10: 581-644 (1988)), and is typically used to treat neoplastic diseases.

15

I. Architecture of the Pin1 Active Site

In copending application (USSN 10/379,115), entitled, “Methods for Designing Specific Inhibitors for Pin1 Proline Isomerase and Pin1 Related
20 Molecules,” filed March 1, 2003 , and expressly incorporated by reference, a method of defining the interaction template and specificity template of Pin1 is disclosed. Through the use of X-ray crystallography and co-crystals of Pin1 polypeptides with molecules bound in the active site, the regions of the active site of Pin1, and PRTP, are defined. Five regions of the active are shown to exist. The five regions are: the
25 phosphate binding pocket; the cysteine/serine valley; the hydrophobic pocket; the substrate entry groove; and lip region. The entire copending application is expressly incorporated by reference herein and the description pertaining to the Pin1 active site is reiterated herein.

30 II. Methods of Treating Pin1-Associated, or PRTP-associated Disorders

In one aspect, the invention includes methods of treatment using a MSPCIT. Enzymes of the Pin1/parvulin class of PPIases are known to be essential for mitosis. Such enzymes have been identified in bacteria, fungi, insect and mammalian cells.

Thus the compounds of the invention are useful for the treatment of a wide variety of disorders involving mitosis and cell proliferation.

A. Pin-Associated States

5 Accordingly, the term "Pin1-associated state" as used herein includes a disorder or a state (e.g., a disease state) that is associated with abnormal cell growth, abnormal cell proliferation, or aberrant levels of Pin1 marker. Pin1-associated states include states resulting from an elevation of cyclin D1 and/or Pin1. Pin1-associated state also includes states resulting from an elevation in the phosphorylation level of c-
10 Jun, particularly phosphorylation of c-Jun on S^{63/73}-P and/or from an elevation in the level of c-Jun amino terminal kinases (JNKs) present in a cell. Pin1-associated states further include states caused by DNA damage, an oncogenic protein (*i.e.*, Ha-Ras), loss of or reduced expression of a tumor suppressor (*i.e.*, Brca1), and/or growth factors.

15 As used herein, the term "abnormal cell growth" is intended to include cell growth which is undesirable or inappropriate. Abnormal cell growth also includes proliferation which is undesirable or inappropriate (*e.g.*, unregulated cell proliferation or undesirably rapid cell proliferation). Abnormal cell growth can be benign and result in benign masses of tissue or cells, or benign tumors. Many art-recognized
20 conditions are associated with such benign masses or benign tumors including diabetic retinopathy, retrolental fibrioplasia, neovascular glaucoma, psoriasis, angiofibromas, rheumatoid arthritis, hemangiomas, and Kaposi's sarcoma. Abnormal cell growth can also be malignant and result in malignancies, malignant masses of tissue or cells, or malignant tumors. Many art-recognized conditions and disorders
25 are associated with malignancies, malignant masses, and malignant tumors.

 "Neoplasia" or "neoplastic transformation" is the pathologic process that results in the formation and growth of a neoplasm, tissue mass, or tumor. Such process includes uncontrolled cell growth, including either benign or malignant tumors. Neoplasms include abnormal masses of tissue, the growth of which exceeds
30 and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change. Neoplasms may show a partial or complete lack of structural organization and functional coordination with the normal tissue, and usually form a distinct mass of tissue. One cause of neoplasia is dysregulation of the cell cycle machinery.

Neoplasms tend to grow and function somewhat independently of the homeostatic mechanisms which control normal tissue growth and function. However, some neoplasms remain under the control of the homeostatic mechanisms which control normal tissue growth and function. For example, some neoplasms are estrogen sensitive and can be arrested by anti-estrogen therapy. Neoplasms can range in size from less than 1 cm to over 6 inches in diameter. A neoplasm even 1 cm in diameter can cause biliary obstructions and jaundice if it arises in and obstructs the ampulla of water.

Neoplasms tend to morphologically and functionally resemble the tissue from which they originated. For example, neoplasms arising within the islet tissue of the pancreas resemble the islet tissue, contain secretory granules, and secrete insulin. Clinical features of a neoplasm may result from the function of the tissue from which it originated. For example, excessive amounts of insulin can be produced by islet cell neoplasms resulting in hypoglycemia which, in turn, results in headaches and dizziness. However, some neoplasms show little morphological or functional resemblance to the tissue from which they originated. Some neoplasms result in such non-specific systemic effects as cachexia, increased susceptibility to infection, and fever.

By assessing the histologic and others features of a neoplasm, it can be determined whether the neoplasm is benign or malignant. Invasion and metastasis (the spread of the neoplasm to distant sites) are definitive attributes of malignancy. Despite the fact that benign neoplasms may attain enormous size, they remain discrete and distinct from the adjacent non-neoplastic tissue. Benign tumors are generally well circumscribed and round, have a capsule, and have a grey or white color, and a uniform texture. By contrast, malignant tumors generally have fingerlike projections, irregular margins, are not circumscribed, and have a variable color and texture. Benign tumors grow by pushing on adjacent tissue as they grow. As the benign tumor enlarges it compresses adjacent tissue, sometimes causing atrophy. The junction between a benign tumor and surrounding tissue may be converted to a fibrous connective tissue capsule allowing for easy surgical remove of benign tumors. By contrast, malignant tumors are locally invasive and grow into the adjacent tissues usually giving rise to irregular margins that are not encapsulated making it necessary to remove a wide margin of normal tissue for the surgical removal of malignant tumors. Benign neoplasms tend to grow more slowly than malignant tumors. Benign

neoplasms also tend to be less autonomous than malignant tumors. Benign neoplasms tend to closely histologically resemble the tissue from which they originated. More highly differentiated cancers, cancers that resemble the tissue from which they originated, tend to have a better prognosis than poorly differentiated cancers.

- 5 Malignant tumors are more likely than benign tumors to have aberrant functions (i.e. the secretion of abnormal or excessive quantities of hormones).

The histological features of cancer are summarized by the term “anaplasia.” Malignant neoplasms often contain numerous mitotic cells. These cells are typically abnormal. Such mitotic aberrations account for some of the karyotypic abnormalities
10 found in most cancers. Bizarre multinucleated cells are also seen in some cancers, especially those which are highly anaplastic. “Dysplasia” refers to a pre-malignant state in which a tissue demonstrates histologic and cytologic features intermediate between normal and anaplastic. Dysplasia is often reversible.

“Anaplasia” refers to the histological features of cancer. These features
15 include derangement of the normal tissue architecture, the crowding of cells, lack of cellular orientation termed dyspolarity, cellular heterogeneity in size and shape termed “pleomorphism.” The cytologic features of anaplasia include an increased nuclear-cytoplasmic ratio (nuclear-cytoplasmic ratio can be over 50% for malignant cells), nuclear pleomorphism, clumping of the nuclear chromatin along the nuclear
20 membrane, increased staining of the nuclear chromatin, simplified endoplasmic reticulum, increased free ribosomes, pleomorphism of mitochondria, decrease in size and number of organelles, enlarged and increased numbers of nucleoli, and sometimes the presence of intermediate filaments.

As used herein, the term “cancer” includes a malignancy characterized by
25 deregulated or uncontrolled cell growth, for instance carcinomas, sarcomas, leukemias, and lymphomas. The term “cancer” includes primary malignant tumors (e.g., those whose cells have not migrated to sites in the subject’s body other than the site of the original tumor) and secondary malignant tumors (e.g., those arising from metastasis, the migration of tumor cells to secondary sites that are different from the
30 site of the original tumor).

The term “carcinoma” includes malignancies of epithelial or endocrine tissues, including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostate carcinomas, endocrine system carcinomas, melanomas, choriocarcinoma, and

carcinomas of the cervix, lung, head and neck, colon, and ovary. The term “carcinoma” also includes carcinosarcomas, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An “adenocarcinoma” refers to a carcinoma derived from glandular tissue or a tumor in which the tumor cells form recognizable glandular structures.

The term “sarcoma” includes malignant tumors of mesodermal connective tissue, *e.g.*, tumors of bone, fat, and cartilage.

The terms “leukemia” and “lymphoma” include malignancies of the hematopoietic cells of the bone marrow. Leukemias tend to proliferate as single cells, whereas lymphomas tend to proliferate as solid tumor masses. Examples of leukemias include acute myeloid leukemia (AML), acute promyelocytic leukemia, chronic myelogenous leukemia, mixed-lineage leukemia, acute monoblastic leukemia, acute lymphoblastic leukemia, acute non-lymphoblastic leukemia, blastic mantle cell leukemia, myelodysplastic syndrome, T cell leukemia, B cell leukemia, and chronic lymphocytic leukemia. Examples of lymphomas include Hodgkin’s disease, non-Hodgkin’s lymphoma, B cell lymphoma, epitheliotropic lymphoma, composite lymphoma, anaplastic large cell lymphoma, gastric and non-gastric mucosa-associated lymphoid tissue lymphoma, lymphoproliferative disease, T cell lymphoma, Burkitt’s lymphoma, mantle cell lymphoma, diffuse large cell lymphoma, lymphoplasmacytoid lymphoma, and multiple myeloma.

For example, the therapeutic methods of the present invention can be used to treat many kinds of cancers (*e.g.*, oligodendroglioma, astrocytoma, glioblastomamultiforme, cervical carcinoma, endometriod carcinoma, endometrium serous carcenoma, ovary endometroid cancer, ovary Brenner tumor, ovary mucinous cancer, ovary serous cancer, uterus carcinosarcoma, breast lobular cancer, breast ductal cancer, breast medullary cancer, breast mucinous cancer, breast tubular cancer, thyroid adenocarcinoma, thyroid follicular cancer, thyroid medullary cancer, thyroid papillary carcinoma, parathyroid adenocarcinoma, adrenal gland adenoma, adrenal gland cancer, pheochromocytoma, colon adenoma mild displasia, colon adenoma moderate displasia, colon adenoma severe displasia, colon adenocarcinoma, esophagus adenocarcinoma, hepatocellular carcinoma, mouth cancer, gall bladder adenocarcinoma, pancreatic adenocarcinoma, small intestine adenocarcinoma, stomach diffuse adenocarcinoma, prostate (hormone-refract), prostate (untreated), kideny chromophobic carcinoma, kidney clear cell carcinoma, kidney oncocyoma,

kideny papillary carcinoma, testis non-seminomatous cancer, testis seminoma, urinary bladder transitional carcinoma, lung adenocarcinoma, lung large cell cancer, lung small cell cancer, lung squamous cell carcinoma, Hodgkin lymphoma, MALT lymphoma, non-hodgkins lymphoma (NHL) diffuse large B, NHL, thymoma, skin malignant melanoma, skin basolioma, skin squamous cell cancer, skin merkel zell cancer, skin benign nevus, lipoma, liposarcoma abnormal cell growth. Specifically, Pin1 has been shown to be overexpressed in the tumor types disclosed in Table 1.

The language "inhibiting undesirable cell growth" is intended to include the inhibition of undesirable or inappropriate cell growth. The inhibition is intended to include inhibition of proliferation including rapid proliferation. For example, the cell growth can result in benign masses or the inhibition of cell growth resulting in malignant tumors. Examples of benign conditions which result from inappropriate cell growth or angiogenesis are diabetic retinopathy, retrolental fibrioplasia, neovascular glaucoma, psoriasis, angiofibromas, rheumatoid arthritis, hemangiomas, Karposi's sarcoma, and other conditions or dysfunctions characterized by dysregulated endothelial cell division.

"Inhibiting tumor growth" or "inhibiting neoplasia" is intended to include the prevention of the growth of a tumor in a subject or a reduction in the growth of a pre-existing tumor in a subject. The inhibition also can be the inhibition of the metastasis of a tumor from one site to another. In particular, the language "tumor" is intended to encompass both in vitro and in vivo tumors that form in any organ or body part of the subject. The tumors preferably are tumors sensitive to the fredericamycin A compounds of the present invention. Examples of the types of tumors intended to be encompassed by the present invention include those tumors associated with breast cancer, skin cancer, bone cancer, prostate cancer, liver cancer, lung cancer, brain cancer, cancer of the larynx, gallbladder, esophagus, pancreas, rectum, parathyroid, thyroid, adrenal, neural tissue, head and neck, colon, stomach, bronchi, kidneys. Specifically, the tumors whose growth rate is inhibited by the present invention include basal cell carcinoma, squamous cell carcinoma of both ulcerating and papillary type, metastatic skin carcinoma, osteo sarcoma, Ewing's sarcoma, veticulum cell sarcoma, myeloma, giant cell tumor, small-cell lung tumor, gallstones, islet cell tumor, primary brain tumor, acute and chronic lymphocytic and granulocytic tumors, hairy-cell tumor, adenoma, hyperplasia, medullary carcinoma, pheochromocytoma, mucosal neuromas, intestinal ganglioneuromas, hyperplastic corneal nerve tumor,

marfanoid habitus tumor, Wilm's tumor, seminoma, ovarian tumor, leiomyomater tumor, cervical dysplasia and in situ carcinoma, neuroblastoma, retinoblastoma, soft tissue sarcoma, malignant carcinoid, topical skin lesion, mycosis fungoide, rhabdomyosarcoma, Kaposi's sarcoma, osteogenic and other sarcoma, malignant

5 hypercalcemia, renal cell tumor, polycythemia vera, adenocarcinoma, glioblastoma multiforma, leukemias, lymphomas (i.e. maglinant lymphomas, mantle cell lymphoma), malignant melanomas, multiple myeloma, epidermoid carcinomas, and other carcinomas and sarcomas.

The language "chemotherapeutic agent" includes chemical reagents that

10 inhibit the growth of proliferating cells or tissues wherein the growth of such cells or tissues is undesirable. Chemotherapeutic agents are well known in the art (see *e.g.*, Gilman A.G., *et al.*, *The Pharmacological Basis of Therapeutics*, 8th Ed., Sec 12:1202-1263 (1990)), and are typically used to treat neoplastic diseases. Examples of chemotherapeutic agents include: bleomycin, docetaxel (Taxotere), doxorubicin,

15 edatrexate, etoposide, finasteride (Proscar), flutamide (Eulexin), gemcitabine (Gemzar), goserelin acetate (Zoladex), granisetron (Kytril), irinotecan (Campto/Camptosar), ondansetron (Zofran), paclitaxel (Taxol), pegaspargase (Oncaspar), pilocarpine hydrochloride (Salagen), porfimer sodium (Photofrin), interleukin-2 (Proleukin), rituximab (Rituxan), topotecan (Hycamtin), trastuzumab

20 (Herceptin), tretinoin (Retin-A), Triapine, vincristine, and vinorelbine tartrate (Navelbine).

Additional cell proliferative disorders contemplated for treatment using the compounds and methods of the invention include fibrotic disorders, non-neoplastic growths such as benign prostatic hypertrophy, endometriosis, psoriasis, and the like.

25 Fibrotic disorders are generally characterized by inappropriate overproliferation of non-cancerous fibroblasts. Examples include fibromyalgia, fibrosis (systic, hepatic, idopathic pulmonary, pericardial, and the like), cardiac fibromas, fibromuscular hyperplasia, restenosis, athersclerosis, fibromyositis, and the like.

30 The MSPCIT molecules of the invention are additionally useful in inhibiting mitosis in pathogenic organisms and are therefore useful for treating infectious diseases. Particular infectious diseases treatable by the methods disclosed herein include bacterial infections and fungal infections.

Bacterial infections contemplated for treatment using the compounds of the invention include infections caused by both gram-positive and gram-negative bacteria, including infections caused by *Staphylococcus*, *Clostridium*, *Streptococcus*, *Enterococcus*, *Diplococcus*, *Hemophilus*, *Neisseria*, *Erysipelothricosis*, *Listeria*,
 5 *Bacillus*, *Salmonella*, *Shigella*, *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Morganella*, *Providencia*, *Yersinia*, *Camphylobacter*, *Myobacteria*, and the like. Infection by such organisms causes a wide variety of disorders including pneumonia, diarrhea and dysentery, anthrax, rheumatic fever, toxic shock syndrome, mastoiditis, meningitis, gonorrhea, typhoid fever, gastroenteritis, brucellosis, cholera,
 10 bubonic plague, tetanus, tuberculosis, Lyme disease, and the like.

Fungal infections contemplated for treatment using the compounds of the invention include fungal infections, dermatophytoses and fungal infections of the genito-urinary tract. Systemic fungal infections include those caused by *Histoplasma*, *Coccidioides*, *Cryptococcus*, *Blastomyces*, *Paracoccidioides*, *Candida*, *Aspergillus*,
 15 *Nocardia*, *Sporothrix*, *Rhizopus*, *Absidia*, *Mucor*, *Hormodendrum*, *Phialophora*, *Rhinosporidium*, and the like. Dermatophyte infections include those caused by *Microsporum*, *Trichophyton*, *Epidermophyton*, *Candida*, *Pityrosporum*, and the like. Fungal disorders of the genito-urinary tract include infections caused by *Candida*, *Cryptococcus*, *Aspergillus*, *Zygomycodoides*, and the like. Infection by such
 20 organisms causes a wide variety of disorders such as ringworm, thrush, San Joaquin fever or Valley fever, Gilchrist's disease, and the like. These infections can be particularly serious, and even fatal, in patients with a depressed immune system such as organ transplant recipients and persons with acquired immunodeficiency syndrome (AIDS).

25

B. Administration of a MSPCIT

The term "subject" is intended to include living organisms, e.g., prokaryotes and eukaryotes. Examples of subjects include mammals, e.g., humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals.
 30 Most preferably the subject is a human.

The language "effective amount" of the compound is that amount necessary or sufficient to treat or prevent a Pin1 associated state, or a PRTP associated state, e.g. prevent the various morphological and somatic symptoms of a Pin1 associated state. In an example, an effective amount of a Pin1 inhibitor of the invention is the amount

sufficient to inhibit undesirable cell growth in a subject. In another example, an effective amount of the Pin1 inhibitor compound is the amount sufficient to reduce the size of a pre-existing benign cell mass or malignant tumor in a subject. The effective amount can vary depending on such factors as the size and weight of the subject, the type of illness, or the particular Pin1 binding compound. For example, the choice of the Pin1 inhibitor compound can affect what constitutes an "effective amount". One of ordinary skill in the art would be able to study the aforementioned factors and make the determination regarding the effective amount of the Pin1 binding compound without undue experimentation. In one possible assay, an effective amount of a Pin1 inhibitor compound can be determined by assaying for the expression of cyclin D1 and determining the amount of the Pin1 inhibitor compound sufficient to reduce the levels of cyclin D1 to that associated with a non-cancerous state.

The regimen of administration can affect what constitutes an effective amount. The Pin1 inhibitor compound can be administered to the subject either prior to or after the onset of a Pin1 associated state. Further, several divided dosages, as well as staggered dosages, can be administered daily or sequentially, or the dose can be continuously infused, or can be a bolus injection. Further, the dosages of the Pin1 inhibitor(s) can be proportionally increased or decreased as indicated by the exigencies of the therapeutic or prophylactic situation.

The term "treated," "treating" or "treatment" includes the diminishment or alleviation of at least one symptom associated or caused by the state, disorder or disease being treated. For example, treatment can be diminishment of one or several symptoms of a disorder or complete eradication of a disorder.

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical composition.

The language "pharmaceutical composition" includes preparations suitable for administration to mammals, e.g., humans. When the compounds of the present invention are administered as pharmaceuticals to mammals, e.g., humans, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The phrase "pharmaceutically acceptable carrier" is art recognized and includes a pharmaceutically acceptable material, composition or vehicle, suitable for administering compounds of the present invention to mammals. The carriers include liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically acceptable antioxidants include: water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, α -tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical, transdermal, buccal, sublingual, rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form

and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range
5 from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and,
10 optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the
15 form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each
20 containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed
25 with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; humectants, such as glycerol; disintegrating agents, such as agar-agar,
30 calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; solution retarding agents, such as paraffin; absorption accelerators, such as quaternary ammonium compounds; wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; absorbents, such as kaolin and bentonite clay; lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene

glycols, sodium lauryl sulfate, and mixtures thereof; and coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluent commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene

glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert dilutents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the active compound in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its

rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral administration is preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into

pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compound of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous and subcutaneous doses of the compounds of this invention for a patient, when used for the indicated analgesic effects, will range from about 0.0001 to about 100 mg per kilogram of body weight per day, more preferably from about 0.01 to about 50 mg per kg per day, and still more preferably from about 1.0 to about 100 mg per kg per day. An effective amount is that amount treats a Pin1 associated state. If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

III. Modulators of Pin1 and P RTP

The crystallized Pin1 R14 A and Pin1 R14A-inhibitor complexes have provided structural information that has enabled identification of the regions of the Pin1 polypeptide involved in the molecular interaction with Pin1 modulators, thereby providing methods for identifying and designing specific modulators of Pin1. Based on the structural elucidation of Pin1, residues in the active site have been identified. The MSPCIT molecules of the present invention interact covalently with residues found in the active site. Further binding specificity and affinity of MSPCIT molecules is conferred through interactions with the specific areas of the active site as described herein.

The Pin1 polypeptide consists of two structural domains organized around a hydrophobic cavity. The N-terminal WW domain, defined as residues 1-39 and a C-terminal proline isomerase domain, defined as residues 45-163. The WW domain is a three-stranded anti-parallel β -sheet. The C-terminal proline isomerase domain is a 4 stranded anti-parallel β -sheet surrounded by 4 α helices (*e.g.*, Ranganathan, *et al.* Cell 89:875-886, 1997; Verdecia *et al.*, Nat Struct Biol 7(8):639-43, 2000).

Based on the comparison of the structural coordinates of the crystals of Pin1 R14A and co-complexes, regions of the active site involved in key interactions which define Pin1 enzyme activity and specificity were elucidated. These regions include the following:

The “hydrophobic pocket” is composed of residues His59, Leu61, Leu122, Phe125, Met130, Gln131, Pro133, Phe134, Thr152, His157 of Pin1 (SEQ ID NO.1). This pocket binds the hydrophobic side chain of the substrate proline.

Residues Leu61, Cys113, and Ser154 in the active site are here defined as a “cysteine/serine valley”, residing between the “hydrophobic pocket” and the “phosphate binding pocket”. The isomerized peptide bond of an alanine-proline dipeptide was found to be located in this region.

The third region is the “phosphate binding pocket” consisting of a region of high localized positive charge and is defined with amino acids Lys63, Ser67, Arg68, Arg69, Pro70, and Ser154. This region is part of the specificity loop (amino acids 64-80) for the phosphate-specific recognition by Pin1 of phosphorylated serine/threonine peptide substrates which are uniquely recognized and isomerized the Pin1 family of proline isomerases, in contrast to FK506 binding proteins and cyclophilins.

A fourth region is the “substrate entry groove” as defined by the following amino acids: Lys63, Arg69, Ser71, Ser72, Trp73, Arg74, Gln75, Glu76, Asp112, Cys113, Ser114.

A fifth region is the “lip regions” as defined by the residues that surround the active site cavity. In one embodiment these lip regions contain residues that are within 10 Å of the active site cavity. In one particular embodiment, this lip region is defined by, but not limited to, residues Arg54, Arg56, His64, Ser65, Gln66, Lys77, Ile78, Thr79, Ser115, Lys117, Ala118, Gly123, Ala124, Phe125, Ser126, Arg127, Gly128, Gln129, Pro133, Glu135, Lys132, Phe151, Asp153, Gly155, and Ile156.

Accordingly, in another aspect, the invention provides a method of designing specific inhibitors of Pin1 isomerase activity based on the ability of molecules to bind to one or more of the defined regions of the Pin1 proline isomerase active site in addition to interacting covalently with at least one residue in the active site.

In one embodiment, the method uses the structural coordinates of Pin1 to design compounds that bind to at least one of the regions of the active site in addition to making a covalent interaction in the active site. In certain embodiments, the compounds are designed to bind at least one, preferably to at least two, more preferably at least three, more preferably at least four and most preferably all five of the regions of the active site in addition to covalently interacting with at least one residue in the active site.

In a further embodiment, potential MSPCIT can be analyzed according to the methods of the invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art. In this embodiment, potential modulators are identified for Pin1 modulatory. Once potential inhibitors are identified, and their structures determined, further optimization can be carried out by computational analyses using the structure coordinates of the Pin1 R14A and Pin1R14A-co-complexes described herein. Alternatively, further optimization can be carried out by determining the structural coordinates of crystallized co-complexes of the potential inhibitor and Pin1 R14A using the methods described herein.

Various combinatorial libraries that can be used in the methods of the invention include, but are not limited to: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is

limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) *Anticancer Drug Des.* 12:145).

Further, examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

The potential inhibitory effect of a compound can be further analyzed prior to its actual synthesis and testing by use of computer modeling techniques using the structural coordinates of the Pin1 R14A and Pin1 R14A-inhibitor co-complexes described herein. If the computer modeling indicates a strong interaction, the molecule can then be synthesized using standard methods known to those skilled in the chemical arts, and then tested for its ability to inhibit Pin1 isomerase activity using the assays set forth herein.

An inhibitory or other binding compound of Pin1 may be computationally evaluated and designed by means of a series of steps in which chemical entities or fragments are screened and selected for their ability to associate with the individual active site regions or other areas of Pin1.

In other embodiments of the method of the invention, potential inhibitory compounds can be examined for their ability to associate with Pin1 and more particularly with the five regions of the Pin1 active site. This process can involve visual inspection of, for example, the active site on the computer screen based on the coordinates of the Pin1 R14A and Pin1 R14A-inhibitor complex. Selected compounds or chemical moieties can then be positioned in a variety of orientations, or docked, within an individual region of the Pin1 active site as defined herein. Docking can be

accomplished using software such as Quanta and SYBYL, followed by energy minimization and molecular dynamics with standard molecular mechanics forcefields, such as CHARMM and AMBER.

Specialized computer programs that can also be used in the process of selecting compounds or chemical entities include:

1. SYBYL Available from Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA
2. UNITY Available from Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA
3. FlexX Available from Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA
4. GRID (Goodford, P. J., "A Computational Procedure for Determining Energetically Favorable Binding Sites on Biologically Important Macromolecules", J. Med. Chem., 28, pp. 849-857 (1985)). GRID is available from Oxford University, Oxford, UK.
5. MCSS (Miranker, A. and M. Karplus, "Functionality Maps of Binding Sites: A Multiple Copy Simultaneous Search Method." Proteins: Structure. Function and Genetics, 11, pp. 29-34 (1991)). MCSS is available from Molecular Simulations, Burlington, Mass.
6. AUTODOCK (Goodsell, D. S. and A. J. Olsen, "Automated Docking of Substrates to Proteins by Simulated Annealing", Proteins: Structure. Function, and Genetics, 8, pp. 195-202 (1990)). AUTODOCK is available from Scripps Research Institute, La Jolla, Calif.
7. DOCK (Kuntz, I. D. et al., "A Geometric Approach to Macromolecule-Ligand Interactions", J. Mol. Biol., 161, pp. 269-288 (1982)). DOCK is available from University of California, San Francisco, Calif.

Once suitable compounds or chemical moieties have been selected, they can be assembled into a single compound or inhibitor. Assembly may be proceed by visual inspection of the relationship of the compounds or moieties to each other on the three-dimensional image displayed on a computer screen in relation to the structure coordinates of Pin1, Pin1 R14A and/or Pin1 R14A-inhibitor co-complexes. This could then be followed by manual model building using software such as Quanta or SYBYL.

Other useful programs to aid one of skill in the art in connecting the individual compounds or chemical entities include:

1. CAVEAT (Bartlett, P. A. et al, "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules". In "Molecular Recognition in Chemical and Biological Problems", Special Pub., Royal Chem. Soc., 78, pp. 182-196 (1989)). CAVEAT is available from the University of California, Berkeley, Calif.

2. 3D Database systems such as MACCS-3D (MDL Information Systems, San Leandro, Calif.). This area is reviewed in Martin, Y. C., "3D Database Searching in Drug Design", J. Med. Chem., 35, pp. 2145-2154 (1992)).

3. HOOK (available from Molecular Simulations, Burlington, Mass.).

In other embodiments, Pin1 inhibitors can be designed as a whole or "de novo" using either an empty active site or optionally including some portion(s) of a known inhibitor(s), e.g., PIN-051 and/or PIN-077, as described herein. Programs which can aid in these methods include:

1. LUDI (Bohm, H.-J., "The Computer Program LUDI: A New Method for the De Novo Design of Enzyme Inhibitors", J. Comp. Aid. Molec. Design, 6, pp. 61-78 (1992)). LUDI is available from Biosym Technologies, San Diego, Calif.

2. LEGEND (Nishibata, Y. and A. Itai, Tetrahedron, 47, p. 8985 (1991)). LEGEND is available from Molecular Simulations, Burlington, Mass.

3. LeapFrog (available from Tripos Associates, St. Louis, Mo.).

Other molecular modeling techniques may also be employed in accordance with this invention. See, e.g., Cohen, N. C. et al., "Molecular Modeling Software and
5 Methods for Medicinal Chemistry", J. Med. Chem., 33, pp. 883-894 (1990). See also, Navia, M. A. and M. A. Murcko, "The Use of Structural Information in Drug Design", Current Opinions in Structural Biology, 2, pp. 202-210 (1992).

Once a compound has been designed or selected by the above methods, the efficiency with which that compound inhibits Pin1 can be tested and optimized by
10 computational evaluation. For example, a compound that has been designed or selected to function as an Pin1-inhibitor must also preferably traverse a volume not overlapping that occupied by the active site when it is bound to the native substrate. An effective Pin1 inhibitor must preferably demonstrate a relatively small difference in energy between its bound and free states (i.e., a small deformation energy of
15 binding).

A MSPCIT can be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target enzyme. Such non-complementary (e.g., electrostatic) interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions. Specifically, the sum of all
20 electrostatic interactions between the inhibitor and the enzyme when the inhibitor is bound to Pin1 preferably make a neutral or favorable contribution to the enthalpy of binding.

Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interaction. Examples of programs designed for
25 such uses include: Gaussian 92, revision C, M. J. Frisch, Gaussian, Inc., Pittsburgh, Pa.; AMBER, version 4.0, P. A. Kollman, University of California at San Francisco; QUANTA/CHARMM, Molecular Simulations, Inc., Burlington, Mass.; and Insight II/Discover (Biosym Technologies Inc., San Diego, Calif.). These programs may be implemented, for instance, using a Silicon Graphics workstation, IRIS 4D/35 or IBM
30 RISC/6000 workstation model 550. Other hardware systems and software packages will be known to those skilled in the art.

Once a Pin1 inhibitor has been optimally selected or designed, as described herein, substitutions can then be made in some of its atoms or side groups in order to improve or modify its binding properties, again using the information provided by the

interaction and specificity templates to identify regions amiable to modification. Generally, initial substitutions are conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. It should, of course, be understood that components known in the art to alter
5 conformation should be avoided. Such substituted chemical compounds may then be analyzed for efficiency of fit to Pin1 by the same computer methods described in detail, above.

The interaction and/or specificity template also can be used to screen for and/or chemically optimize small molecules or chemical entities for Pin1 modulating
10 activity, e.g., inhibiting activity. These molecules can be molecules previously identified as having Pin1 activity using a biological assay and/or can be molecules suspected of having such activity based on their structure. Examples of Pin1 modulating compounds include compounds described in WO 03074550 A2, WO 03073999 A2, WO 03074497 A1, WO 04028535A1, WO 03074001A2, WO
15 03074002A2, and U.S. Provisional Application No: 60/537,171, filed January 16, 2004, entitled "Pin1-Modulating Compounds and Methods of Use Thereof." The compounds described in these copending applications can be altered such that they have the ability to covalently interact with residues in the active site of Pin1.

Further examples of compounds that covalently modify Pin1 can be found in
20 U.S. Serial Number 10/XXX,XXX, entitled "*PHOTOCHEMOTHERAPEUTIC COMPOUNDS FOR USE IN TREATMENT OF PIN1-ASSOCIATED STATES*," filed on even date herewith and expressly incorporated herein by reference.

In certain embodiments of the invention, a modulator of Pin1 activity, or PRTP activity, is administered in combination with other agents, or in conjunction
25 with another, complementary treatment regime. For example, in one embodiment, MSPCIT is used to treat a cellular proliferation, growth, differentiation, and/or migration disorder. Accordingly, modulation of Pin1 activity may be used in conjunction with, for example, another agent or treatment used to treat the disorder, e.g., radiation or conventional chemotherapy.

30 The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent

applications and published patents, cited throughout this application are hereby expressly incorporated by reference. The animal models used throughout the Examples are accepted animal models and the demonstration of efficacy in these animal models is predictive of efficacy in humans.

5

EXAMPLES

Example 1: Tissues with Elevated Pin1 Levels

10 Automated cellular imaging system (ACIS) was used to determine tissues with elevated Pin1 Levels. The data that is presented in Example 1 is from U.S. Patent Application No. 10/071,747, filed February 8, 2002, the entire contents of which are incorporated by reference.

15 Micro-histoarray sections were scanned and images were captured using the automated cellular imaging system (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA) which combines automated microscopy and computerized image processing to analyze of multiple tissues on a single slide. ACIS was used to analyze microarray tissue sections on glass slides stained using a diaminodenzidine chromagen (DAB) and hematoxylin counterstain. Positive
20 staining (brown color) as viewed by light microscope indicates the presence of the protein, and color intensity correlates directly with protein quantity (expression). The ACIS was able to recognize 255 levels of immunohistochemical staining intensity (0-255) and converted these to fractional scores for the selected individual areas. However, the base limit on the threshold for the Generic DAB is
25 pre-set at 50 by the manufacturer because the system is very sensitive. Therefore, any intensity below 50 was treated as 0 in this study. Entire immunostained tissue sections were scanned using the 4 X objective and images were captured using the 10X objective.

30 Calculation of Pin protein expression in human cancers

In this study, intensity scoring and the percent positive scoring (brown area was divided by total area) were used with the entire individual tissue dot selected. The immunohistochemical staining was quantitated without knowledge of a

pathologist's score. All tissue samples were immunostained twice in University of Basel and in Pintex Pharmaceuticals, Inc. and the two data sets were evaluated in Pintex Pharmaceuticals, Inc. The final score was obtained by using the average of two data sets and calculated by the formulation:

5

$$\text{score} = \text{intensity} + (\text{X percent positive staining}).$$

10

$$\frac{\text{The \% of total cases showing elevated levels (over-expression) of Pin 1 = } \frac{\text{[numbers of tumor samples with score larger than the score of the highest normal case)}}{\text{total number of tumor samples}}$$

Results

15 **Table 1:** Pin1 protein over-expression in human tissues microarray

Tumor type	Case number	% of Tumor Cases with Elevated Level of Pin1
<u>Brain tumor (3)</u>	111	
Oligodendroglioma	20	90
Astrocytoma	46	63
Glioblastomamultiforme	45	87
<u>Genecological tumor (13)</u>	372	
Cervical carcinoma	42	81
Endometrium, endometroid carcinoma	46	0
Endometrium, serous carcinoma	13	0
Ovary, endometroid cancer	45	24
Ovary, Brenner tumor	8	63
Ovary mucinous cancer	12	58
Ovary, serous cancer	47	43
Uterus, carcinosarcoma	6	100

Breast, lobular cancer	36	56
Breast, ductal cancer	47	47
Breast, medullary cancer	24	29
Breast, mucinous cancer	24	29
Breast tubular cancer	22	9
<u>Endocrine tumor (8)</u>	213	
Thyroid adenocarcinoma	42	29
Thyroid follicular cancer	49	41
Thyroid medullary cancer	8	100
Thyroid papillary car	36	22
Parathyroid, adenocarcinoma	28	21
Adrenal gland adenoma	15	0
Adrenal gland cancer	6	33
Pheochromocytoma	29	0
<u>Digestive tract tumor (11)</u>	411	
Colon adenoma mild displasia	47	21
Colon adenoma moderate displasia	47	17
Colon adenoma severe displasia	49	14
Colon adenocarcinoma	43	2
Esophagus adenocarcinoma	43	30
Hepatocellular carcinoma	34	62
Mouth cancer	46	93
Gall bladder adenocarcinoma	28	14
Pancreatic adenocarcinoma	43	2
Small intestine adenocarcinoma	10	0
Stomach diffuse adenocarcinoma	21	0
<u>Genitourinary tract tumor (9)</u>	381	

Prostate (hormone-refract)	44	59
Prostate (untreated)	47	64
Kidney chromophobic carcinoma	15	0
Kidney clear cell carcinoma	47	0
Kidney oncocytoma	8	0
Kidney papillary carcinoma	44	0
Testis, non-seminomatous cancer	43	2
Testis seminoma	47	2
Urinary bladder transitional carcinoma	86	2
<u>Respiratory tract tumor (4)</u>	184	
Lung, adenocarcinoma	44	27
Lung, large cell cancer	45	42
Lung, small cell cancer	47	57
Lung, squamous cell carcinoma	48	44
<u>Hematological neoplasia (5)</u>	146	
Hodgkin lymphoma	23	0
MALT lymphoma	47	4
NHL, diffuse large B	22	18
NHL, others	30	23
Thymoma	24	8
<u>Skin tumor (5)</u>	178	
Skin, malignant melanoma	44	73
Skin, basolioma	44	39
Skin, squamous cell cancer	39	13
Skin, merkel zell cancer	5	100
Skin benign nevus	46	52

Soft tissue tumor (2)	45	
Lipoma	25	20
Liposarcoma	20	75

5 **Example 2: Covalent Modification by Fred-A and Juglone**

Pin1 was incubated with Fred-A to determine the effect on peptidyl prolyl isomerase activity. This experiment was performed to determine if Fred-A covalently modifies Pin1 with a similar mechanism to Juglone which is known to covalently modify the active site of Pin1 as shown by Henning, L. et.al. (Selective
 10 Inactivation of Parvulin-Like Peptidyl-Prolyl cis/trans Isomerases by Juglone. (1998) Biochemistry 37 5953-5960).

Juglone

A solution containing 7.4 uM Pin1, 100 uM juglone 1% DMF, in 35 mM
 15 hepes pH 7.8 was incubated. At various time points, aliquots were removed and diluted into assay buffer containing 1nM Pin1, 13.5 nM juglone, 0.1 mg/ml BSA, 0.2 mM DTT, and 35 mM Hepes pH 7.8. Pin1 activity was measured by using a protease coupled Pin1 assay.

Aliquots of the incubation mixtures were diluted 7,200 fold into assay buffer
 20 (0.1 mg/mL BSA, 0.2 mM DTT and 35 mM Hepes pH 7.8) to a final Pin1 concentration of 1 nM. 20 uL of a 0.4 mg/mL trypsin solution in 35 mM Hepes pH 7.8 was added to 1.2 mL Pin1 solution. The enzymatic reaction was initiated by the addition of 3 uL the substrate solution (15/mg/mL of Ac-Ala-Ala-(PO₃)Ser-Pro-Arg-pNA dissolved in a solution of 0.5 M LiCl in trifluoroethanol). The reaction progress
 25 was monitored at 390 nm for 8 minutes at 4°C.

Fred-A

A solution containing 7.4 uM Pin1, 200 uM juglone 1% DMF, in 35 mM
 hepes pH 7.8 was incubated. At various time points, aliquots were removed and
 30 diluted into assay buffer containing 1nM Pin1, 27 nM juglone, 0.1 mg/ml BSA, 0.2

mM DTT, and 35 mM Hepes pH 7.8. Pin1 activity was measured as described above for juglone.

The results indicate that Pin1 is modified in a manner consistent with covalent
5 modification of the active site by Fred-A (see figure 2).